The Roles of Gαq and Phospholipase C Beta in Regulating Mechanosensory and Thermosensory Behavior in *Drosophila*

by

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Abstract

Nociception is the ability of sensory neurons to detect thermal, mechanical, or chemical stimuli that can be harmful to an organism and generate a behavioral response. This is a process that has been conserved throughout vertebrate and invertebrate systems including the fruit fly, *Drosophila melanogaster*. G proteins are known to regulate sensory neuron function in many systems, but no specific role has been identified in *Drosophila* nociception. Gαq is a Gα subunit of the heterotrimeric G protein complex that activates Phospholipase C beta (PLC-β), an enzyme responsible for the mediation of the signaling molecules, phosphatidylinositol 4,5-bisphosphate (PIP_2), inositol 1,4,5-trisphosphate (IP_3), and diacylglycerol (DAG). These enzymes are known to regulate the function of many different types of neurons, suggesting that they might have a role in controlling the function of *Drosophila* nociceptors, the sensory neurons that detect noxious stimuli. Transgenic flies with altered *Gαq* and *NorpA* (*PLC-β* in *Drosophila*) signaling were stimulated with thermal and mechanical stimuli and behavioral responses were used to determine sensory neuron function. Gene function of *Gαq* and *NorpA* were reduced in transgenic flies using RNA interference (RNAi), which resulted in a reduced behavioral response to a thermal and mechanical stimulus. *NorpA* and *Gαq* were overexpressed with genetic manipulations that increased the protein level or enzymatic activity of the proteins, which generally yielded an increased response to mechanical stimuli. These results suggest that Gαq and PLC proteins positively regulate the function of *Drosophila* sensory neurons and that this signaling pathway is important for thermal and mechanical nociception. These experiments identify the roles of *PLC* and *Gαq* as necessary for normal nociception and provide a foundation for future experiments that will identify the cellular/molecular mechanisms of how these enzymes function in *Drosophila* nociceptor neurons.

Keywords: *Drosophila*, Nociception, Pain, G-Protein, *Gαq*, Phospholipase C, *NorpA*, RNAi, Overexpression, Gain of Function, Neurons, Mechanosensation, Thermosensation

Table of Abbreviations

Abbreviation	Description
AR	ankyrin repeats
AT_1R	angiotensin II receptor, type 1
BRET	bioluminescence resonance energy transfer
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DAG	diacylglycerol
DEG/ENaC	degenerin/epithelial Na^+ channel
DI	distilled
dTKR	*Drosophila* tachykinin receptor
GDP	guanosine 5'-diphosphade
GoF	gain of function
GPCR	G protein-coupled receptor
GTP	guanosine 5'-triphosphate
IOM	Institute of Medicine
IP_3	inositol 1,4,5-triphosphate
mdIV neurons	multidentritic class IV neurons
NEL	nocifensive escape locomotion
NorpA	phospholipase C beta
Para	*Paralytic* gene
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PNS	peripheral nervous system
ppk	*pickpocket* gene
PUFA	poly unsaturated fatty acid
RISC	ribonucleic acid induced silencing complex
RNAi	ribonucleic acid interference
siRNA	short interfering ribonucleic acid fragments
TAC	transient receptor potential ankyrin cap
TRP	transient receptor potential
UAS	upstream activation sequence

Introduction

The Effects of Pain on Humans

In 2010, the Patient Protection and Affordable Care Act was passed in the United States. This act lead the Institute of Medicine (IOM) to regard chronic pain as a national public health issue. They found that chronic pain effects more than 100 million people and is associated with an annual cost of $635 billion in the United States alone. Management of chronic pain often includes the need for specialized treatment and pain clinics, medications, physical therapy, and invasive techniques such as surgery (Relieving Pain in America: A Blueprint for Transforming Prevention, Care, Education, and Research, 2012). Breivik et al. conducted a study which demonstrated some of the issues that can be associated with the treatment of chronic pain in European Adults. They found that 21 percent of people had been diagnosed with depression due to chronic pain, 19 percent had lost their jobs as a result of chronic pain, and that nearly two-thirds of people with chronic pain were taking prescription medications including the highly addictive, strong opioids (Breivik Collett, Ventafridda, Cohen, & Gallacher, 2006). Howard et al. conducted a study which assessed neuropathic pain, a component of chronic pain in children. They found that chronic neuropathic pain, that is, lasting more than 3 months, occurred in 6 percent of children and adolescents. This pain state was generally the result of cancer/chemotherapy, trauma, genetic disorders, neurological, and metabolic disease or chronic infection (Howard, Wiener, & Walker, 2014). Most of these causes are without a cure and can plague children for a long duration of time. These studies raise awareness for the need to further scientific research in the field and develop more effective treatments and potential cures. In order to effectively do this, it is essential that the mechanisms of pain be identified and studied to establish more effective clinical treatments.

Studying Nociception in Drosophila

Nociception is the ability of sensory neurons to detect potentially harmful thermal, mechanical, or chemical stimuli and generate a behavioral response. Noxious stimuli are detected by nociceptors, a subset of polymodal (able to detect noxious temperature, mechanical, chemical stimuli) somatosensory neurons (Bautista et al., 2006). To identify the mechanism of pain reception, it is necessary to understand the mechanisms at work within nociceptors. The invertebrate, *Drosophila melanogaster,* has been proven to be an effective model in the identification of genes required for nociception (Caldwell, & Tracey, 2010; Brierley et al., 2009). Short life spans, ease of genetic manipulation, a simple nervous system, cost-efficacy, expansive transgenic *Drosophila* line availability, and ease of *Drosophila* stock maintenance make this organism not only an effective but premier model for studying pain (Caldwell, & Tracey, 2010).

Drosophila exhibit a distinct behavior in response to noxious stimuli known as nocifensive escape locomotion (NEL; Caldwell, & Tracey, 2010). NEL in *Drosophila* consists of a distinct cork-screw or barrel-roll around the longitudinal axis of the larvae that is genetically controlled (Bautista et al., 2006; Caldwell, & Tracey, 2010; Robertson, Tsubouchi, & Tracey 2013) and easily scored in behavioral assays. Nocifensive escape locomotion is a behavior that is triggered in response to potentially harmful mechanical, thermal, and chemical stimuli (Brierley et al., 2009; Hwang, Stearns, & Tracey, 2012) making it a reliable indicator of pain reception in *Drosophila* larvae. This behavior is thought to have developed as an evolutionary response to defend larvae from female *Leptopilina boulardi*, a species of parasitoid wasps that utilize a pointed ovipositor to imbed eggs within the larvae of *Drosophila*. The larvae of the wasp then proceed to effectively eat their host from the inside out (Robertson et al., 2013),

making it reasonable that larvae would have developed a defense mechanism to protect against hosting this parasite.

There are 4 distinct classes of multidendritic somatosensory neurons in *Drosophila* larvae, Classes I-IV (A Grueber, Ye, Moore, Jan, L. Y., & Jan, Y. N., 2003). As it pertains to pain, Class I and II neurons are generally associated with actions such as normal locomotion, class III neurons are typically associated with light touch, and multidendritic class IV (mdIV) neurons are responsible for perception of noxious stimuli. The nocifensive escape locomotive response is dependent on the nociceptors known as the mdIV neurons in larvae (Robertson et al., 2013; Hwang et al., 2012). For this study, it is most important to note that NEL provides us with a readout of nociceptor sensitivity. Class IV Multidendritic neurons are part of the peripheral nervous system (PNS) with highly branched dendrites in the periphery. In *Drosophila*, mdIV neurons can have as many as 900 terminal branches per neural cell and are well ordered to cover the epidermis of each larval segment (Grueber, Jan, L. Y., & Jan, Y. N., 2002). Somatosensory neurons have complex cellular transduction pathways with many components that produce the detection of noxious stimuli in *Drosophila* and evoke the NEL behavior.

TRPA1 is an ion channel necessary for nociception

One component of cellular transduction pathways necessary for the perception of pain is a member of the Transient Receptor Potential (TRP) channel family, TRPA1, a nonspecific cation channel that has been well studied in *Drosophila*. There are a number of TRP channels including TRPA, TRPN, TRPL, and TRPV which function in the transduction of noxious stimuli (Tracey, Wilson, Laurent, & Benzer, 2003). The TRPA1 channel was first discovered to have a direct role in human pain syndromes during a genetic investigation in a Colombian family that

had been diagnosed with a heritable familial episodic pain syndrome. TRPA1 in *Drosophila* is thought to be activated by noxious chemicals through the covalent modifications of cysteine residues by electrophiles as well as formation of disulfide bridges (Kremeyer et al., 2010; Macpherson et al., 2007). TRPA1 was shown to play a role in the transduction pathway of chemical irritants that yield inflammatory pain in mice so there is some conservation of *TRPA1* function between invertebrates and mammals (Bautista el at., 2006). Brierley et al. conducted a study which demonstrated that TRPA1 channels are necessary for mechanosensation, the detection of noxious physical stimuli in mice (Brierly et al., 2009). This is consistent with the evolutionary hypothesis of parasitic avoidance but a direct mechanism for how a mechanical stimulus activates TRPA1 channels has not yet been confirmed. There is a hypothesis that Ankyrin repeats (AR), a protein motif composed of two alpha helices and flexible linker region, associated with TRPA1 channels may be the mechanism in which TRP channels are activated by noxious mechanical stimuli. The argument for this is presented by Zhang et al. in a study which demonstrated that *no mechanoreceptor potential C* (*NOMPC*), which codes for a TRP channel is involved in mechanotransduction in *Drosophila* contains 29 ARs. They were able to show that the integrity of ARs, which associate with microtubules, are necessary for the mechanogating of NOMPC channels. This suggests that ARs play a “tethering” role critical to the opening and closing of the NOMPC channel (Zhang et al., 2015). The mechanism of mechanical nociception however, is currently a controversial topic in the field. Hwang et al. conducted a study which demonstrated that Ankyrin repeats are <u>not</u> necessary for mechanical nociception in all TRPA channels, specifically it is not necessary for mechanical nociception in the TRPA Painless channel (Hwang et el., 2012). Clearly, further investigation is required to determine the mechanisms of mechanosensation in TRP family ion channels.

Zong and Bellemer et al. conducted a study which demonstrated that TRPA1 channels were necessary for thermal nociception in *Drosophila.* Specifically, they showed that of the four known TRPA1 isoforms, TRPA1-B and TRPA1-C, do not respond to temperature changes within 15-42°C. They did however find that temperatures between 24-29°C activated the TRPA1-A isoform and temperatures between 34-36°C activated TRPA1-D (Zhong, & Bellemer et. al., 2012). This suggests that TRPA1-A may be more of a basal heat receptor necessary for discrimination of small temperature differences and TRPA1-D is more likely to be involved in the detection of noxious heat. They were then able to begin uncovering the mechanism by which heat is detected in these isoforms, by determining variations in distinct 37-amino acid sequences bound to the end of TRPA1 channels, called the N-terminal TRP Ankyrin Cap (TAC), that are essential for TRPA1 to elicit a thermal response. Additionally, the N-terminal TRP Ankyrin Cap (TAC) on isoforms A and D was shown to affect the threshold for the TRPA1 channel (Zhong, & Bellemer et. al., 2012). This finding suggests a role for the Ankyrin repeats in activation of the TRPA1 channels by temperature.

Painless - Another TRP Channel Shown to be Critical to Nociception

Zong and Bellemer et al. conducted a study which discovered a nociceptive role for the gene, *painless,* which codes for the ion channel known as Painless. Painless is another transient receptor potential (TRP) family channel that like *TRPA1*, is expressed in multidendritic somatosensory neurons and involved in nociceptive signaling. Tracey et al. conducted a study which demonstrated that a variety of genetic loss-of-function experiments reinforced by rescue experiments to demonstrate that, similar to *TRPA1*, *painless* is necessary for the detection of noxious thermal and mechanical stimuli (Tracey et al., 2003). *painless* was later shown to play a

role in chemical nociception as well (Al-Anzi, Tracey, & Benzer, 2006). Unlike TRPA1, the Painless channel does not play a role in the detection of light stimuli, making this channel specific to nociception (Tracey et al., 2003). Sokabe et al. conducted a study which demonstrated that in a heterologous system, *painless*, encodes a Ca^{2+} dependent channel that mediates a substantial Ca^{2+} influx in response to noxious thermal stimuli. They demonstrated Painless is able to detect heat directly using Ca^{2+} ions but not via other cellular pathways indicating that *painless* plays an important role in the detection of noxious heat (Sokabe, Tsujiuchi, Kadowaki, & Tominaga, 2008). In *Drosophila*, nociception assays are often performed on wandering third instar larvae that have developed nervous systems but are still in the larval state. Xu et al. conducted a study that used a nocifensive escape jump response in to test the role of *painless* in adult flies. They found that in adult flies, *painless* also plays a role essential to thermal nociception. This finding was consistent with the thermal nociception studies previously performed on *Drosophila* larvae (Tracey et al., 2003; Sokabe et al., 2008). This study importantly conveyed the role of *painless* to be the same in the larval and adult nociceptors (Xu et al., 2006). Additionally, *painless* has been shown to have more than just a nociceptive function and plays a role in the aversion of unfavorable moisture conditions in larvae (Johnson, & Carder, 2012), and a role in sexual behaviors of adult females (Sakai, Kasuya, Kitamoto, & Aigaki, 2009). This serves as a reminder of the level of complexity involved in intracellular signaling and just how multifaceted biological units can be.

TRPA1 and Painless channels have been shown to play a substantial role in both mechanical and thermal nociception pathways but there are a number of other components involved in each respective pathway that have not been shown to play a role in the other form of nociception. While some components of the signaling transduction pathway involved in thermal

and mechanical nociception have been shown to be shared amongst both pathways, known mechanospecific channels such as NOMPC means these forms of nociception do not operate through identical pathways. In mechanical nociception, there is an entire class of TRP channels, TRPN channels, Pickpocket, a Degenerin/Epithelial Na^+ Channel (DEG/ENaC), and Piezo, a eukaryotic excitatory channel, all play a critical role in mechanosensation in *Drosophila*.

Molecular Mechanisms of Mechanical Nociception

Mechanosensation is most simply described as a transduction pathway that receives a mechanical force and translates that stimulus into an electrical signal that travels from the PNS to the brain (Walker, Willingham, & Zuker, 2000). The cellular machinery required to make this translation from mechanical force to electrical signal is complex and still largely unclear. However, some elements of the signaling transduction pathway of mechanosensation in *Drosophila* have been discovered such as the NOMPC channel. The NOMPC channel, mentioned before, is a member of the TRP channel family but is classified as a TRPN channel rather than a TRPA channel (Fowler, & Montell, 2013). NOMPC tends to be located in mechanosensory organs including the tips of mechanosensory bristles on the proboscis of an adult fly (Walker et al., 2000). The NOMPC channel was shown to be a component of mechanotransduction in the mechanosensory mdIV neurons of *Drosophila* that is likely to be critical to the normal detection of noxious mechanical stimuli (Gong, Wang Q., & Wang Z., 2013). The detection of the physical stimuli is dependent on the 29 Ankyrin Repeats on the N-terminus end of NOMPC which has the largest known number of known ARs of any TRP channel (Zhang et al., 2015). *NOMPC* expressed in the class III neurons has also been shown to play a role in the sensing of light touch in *Drosophila* larvae (Tsubouchi, Caldwell, & Tracey,

2012) showing that *NOMPC* can perform multiple functions depending on the tissue in which it is expressed. It has been made apparent through these studies that NOMPC has a significant function in mechanical nociception but such has failed to be demonstrated in thermal nociception.

Zhong et al. conducted a study which discovered another channel that plays a role in mechanical nociception, Pickpocket. The DEG/ENaC subunit encoded by the gene *pickpocket* (*ppk*) is found in the mdIV neurons. *painless* was shown to be essential to mechanical nociception but not to thermal nociception or optogenetic activation in *Drosophila* larvae. In this study, RNAi was used to knockdown the gene function of *pickpocket* which displayed a defective phenotype in the reception of noxious mechanical stimuli without displaying an abnormal phenotype in the reception of light mechanical stimulus (<30mN). This gives evidence for a nociceptor-specific mechanosensory function for *ppk*, rather than a broad excitatory function of mdIV neurons in *Drosophila* larvae. Because of *pickpocket's* lack of a role in thermal nociception, it was made clear that *ppk* operates in a different manner than the previously mentioned, *painless*. This proves an assertion made earlier, mechanical and thermal nociception are genetically different and thus the pathways responsible for each must be different. Despite being necessary for mechanical nociception in *Drosophila* larvae, *ppk* alone is not sufficient for mechanosensation and likely requires formation of a complex with other DEG/ENaC subunits to yield wild-type mechanotransduction (Zhong, Hwang, & Tracey, 2010).

The third mechanosensory specific component of mechanotransduction in *Drosophila* as identified by Kim et al. in a study which demonstrated that *piezo* (*dpiezo* in *Drosophila*) has been linked to *pickpocket* and shown to play a similar function in the signal transduction pathway involved in detection of noxious stimuli (Kim, Coste, Chadha, Cook, & Patapoutian, 2012).

dpiezo encodes a transmembrane excitatory channel that has been show to play a significant role in mechanical nociception in *Drosophila.* Knockout of *dpiezo* using genomic FLP-FRT recombination knocks down the 31 exons or coding regions of the gene effectively silencing the gene. In these *dpiezo* knockouts they observed that only 34 percent of the *Drosophila* larvae showed a nocifensive response to a 45 mN mechanical stimuli when more than 80 percent of wild-type flies responded to the same stimulus without effecting the larvae's ability to detect a gentle touch stimulus. To make this study more robust, they also showed that a rescue of *dpiezo* using cDNA proved successful in restoring a wild-type phenotype in response to the noxious mechanical stimulus. This shows a significant reduction in mechanosensation and thus establishes the Piezo channel as an important component of mechanical nociception in *Drosophila*. Kim et al. proceeded to make genetic manipulations necessary to observe the interactions that Pickpocket and Piezo have with each other. They made a novel discovery that *ppk*-expressing neurons can elicit a response to noxious stimuli, Pickpocket requires mechanically activated Piezo-dependent electrical currents (Kim et al., 2012). This study suggests that if Pickpocket is dependent on Piezo then these two factors function in the same pathway. Suslak et al. conducted a study which demonstrated that *dpiezo* likely plays the primary role in detection of potentially harmful mechanical stretching of cells. They also found that the function of p*iezo* is not conserved across species as they mention that the Piezo channel is a light touch detector in zebrafish and mice while it plays no role in light touch in *Drosophila* but rather serves as a noxious sensor (Suslak et al., 2015).

G Protein-Coupled Receptors as they Pertain to Mechanosensation

Recently, studies have been done that show a potential link between G protein-coupled receptors (GPCRs) and mechanosensation (Schnitzler, Storch, & Gudermann, 2011; Scholz et al., 2015; Makino et al., 2006; Storch, Schnitzler, & Gudermann, 2012). GPCRs are a large family of receptors that bind with G proteins that hydrolyze guanosine 5'-triphosphate (GTP). GPCRs are a prominent and highly differentiated family of receptors with more than 200 known isoforms found in *Drosophila.* G protein-coupled receptors act as guanine nucleotide exchange factors that activate/inactivate bound heterotrimeric G proteins leading to the activation of a multitude of cell signaling pathways (McCudden, Hains, Kimple, Siderovski, & Willard, 2005). GPCRs are widely conserved amongst most life forms including humans, bacteria, and zebrafish. In these organisms, GPCRs have been shown to bind to a range of different ligands including proteins, hormones, and even detection of light particles. (Hanlon, & Andrew, 2015). GPCRs have specifically been demonstrated to play a critical role in responses to environmental chemicals, hormones, and neurotransmitters that contribute to inflammation, cell proliferation or differentiation, cancer (activation of G proteins such as Ras) and olfactory function (Storch et al., 2012; Hanlon, & Andrew, 2015; McCudden et al., 2005). Due to such a wide range of roles fulfilled by GPCRs it was logical that this group of receptors may play a role in mechanosensation.

Makino et al. conducted a study which demonstrated that that GPCRs played a role in neutrophils (a type of white blood cell) as mechanosensors for fluid shear stress in HL60 cells. They asserted that this mechanism is likely important for the triggering of an immune system response to tissue damage in humans (Makino et al., 2006). This discovery posed the query, if GPCRs played a role in mechanosensation as it pertains to an immune system response, could

they also play a mechanosensory role in nociceptors? Schnitzler et al. were the first to demonstrate the likelihood of this role for GPCRs. They showed that Angiotensin II, Type 1 receptor (AT_1R) in addition to histamine H_1, muscarinic M_5, and vasopressin V_{1A} are all GPCRs that were found to be mechanosensitive in mice. Schnitzler et al. suggests two distinct mechanisms for the direct detection of mechanical stimuli for AT_1R including a "membrane-stretch-induced" mechanism and a "tethered" mechanism. They used bioluminescence resonance energy transfer (BRET) experiments in which they showed that mechanical stimulus directly causes a conformational change in the membrane bound AT_1R resulting in activation of the receptor (Schnitzler et al., 2011). The data produced in the BRET experiment gives evidence to support the proposed AT_1R tethering mechanism. Interestingly, a study published two years later by the same authors identified a ligand-independent mechanically induced conformation for AT_1R that differed from the ligand-dependent conformation furthering the complexity of this GPCR (Storch et al., 2012). These studies helped concretely identify a mechanosensory role for GPCRs but a neuro-mechanosensory role had not yet been identified. Scholz et al. conducted a study which did exactly that in *Drosophila* when they identified Latrophilin/CIRL, an adhesion GPCR- in which a mechanical stimulus as well as a ligand required for activation, is necessary for mechanosensory neuron function in chordotonal neurons in the central nervous system in response to gentle touch. Notably, they also determined that Latrophilin/CIRL interacts with downstream TRP channels which raises a number of questions as to the role of the molecules downstream of GPCRs in thermal and mechanical nociception (Scholz et al., 2015). Schnitzler et al. conducted a study which demonstrated that PLC is likely to play a vital role in mechanotransduction in *Drosophila* but fails to comment on mechanical nociception.

Gαq and NorpA May Play a Role in Thermal and Mechanical Nociception

Despite the apparent role of GPCRs in detection of gentle touch, a role for GPCRs in mechanical nociception within mdIV neurons in *Drosophila* has yet to be identified. Furthermore, if GPCRs play a role in mechanical nociception it is logical that the immediately downstream heterotrimeric G proteins, bound to and activated by the GPCRs may also play a role in mechanical nociception in mdIV neurons. Heterotrimeric G proteins are composed of alpha, beta, and gamma subunits that are activated via exchange of guanosine 5'-diphosphate (GDP) for GTP. When GTP binds the alpha subunit (Gα) dissociates from the beta and gamma dimer. The Gα subunit is then able to interact with its downstream effectors and play a role in many aspects of cellular signaling, when the associated GTP is hydrolyzed to GDP, the Gα subunit is inactivated (McCudden et al., 2005). Additionally, the interaction that GPCRs are believed to have with TRP channels, makes the investigation of G proteins and their role in thermal nociception worth investigating. Gαq and NorpA (a Phospholipase C homologue) have been shown to play a critical role in the Phototransduction pathway in *Drosophila* (Hardie et al., 2002) but did not have any noception-associated roles in previous literature. **Identifying a role for G-proteins, specifically the Gαq subunit, in thermal and mechanical nociception was one of the primary objectives of this study.**

Phospholipase C (PLC, *NorpA* in *Drosophila*) is a regulatory molecule, immediately downstream of G-protein proteins, activated by the Gαq subunit, but upstream of TRPA1 in the TRPA1 signaling pathway. PLC is an enzyme that regulates three main second messengers, phosphatidylinositol 4,5-bisphosphate (PIP_2), inositol 1,4,5-triphosphate (IP_3), and diacylglycerol (DAG), that go on to influence metabolites such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (Fowler et al., 2013). PLC has been shown to

indirectly affect TRPA1 channels in mice through its effect on phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_2 is a secondary metabolite that sequesters intracellular Ca^{2+} release, PLC plays a catalytic role on PIP_2 in which PIP_2 is hydrolyzed into IP3 and Diacylglycerol. The reduction of intracellular PIP_2 reduces the Ca^{2+} sequestering function of the messenger and thus enables an increase in the release of intracellular Ca^{2+} levels. This provides a mechanism in which PIP_2 plays a direct role on the function of known Ca^{2+} channel, TRPA1 (Bessac, & Jordt, (2008). Inositol 1,4,5-triphosphate (IP3) is the molecule formed by the hydrolysis of PIP_2 and has not been shown to play any known role in the activation of TRP channels and consequently unlikely to play a role in mechanical nociception (Fowler et al., 2013). Diacylglycerol (DAG) is another secondary messenger that is influenced by PLC. DAG has been shown to act as an excitatory messenger responsible for channel activation in phototransduction of light in *Drosophila*. The degradation of DAG by DAG Kinase has been shown to be the first step in regeneration of PIP_2, a known antagonist of TRPA1. Additionally, DAG has been shown to release poly unsaturated fatty acids (PUFAs) through interactions with DAG lipase which are believed to play a gating function in TRP channels (Fowler et al., 2013; Hardie et al., 2002). DAG has been shown to activate protein kinase C (PKC) which has been shown to regulate Orco, a known cation channel that functions as an olfactory receptor (Fowler et al., 2013; Sargsyan et al., 2011). These roles of DAG make this molecule one more reason that NorpA (PLC-β) is likely to play a role in nociception in *Drosophila*. Collectively, the previously mentioned channels such as Orco, Painless, Piezo, and TRPA1 are part of the known mechanisms for the signaling transduction pathway for nociception and are potential targets of regulation in *Drosophila*. **The second primary objective of this study was to identify the role that *NorpA* plays in mechanical and thermal nociception in *Drosophila*.**

Methods

Fly husbandry:

The fly stocks used in this study (Table 1) were ordered from Bloomington *Drosophila* Stock Center at Indiana University and raised on standard cornmeal molasses fly food at room temperature. Flies were transferred to new vials with fresh food every 17 days.

Table of stocks used:

Table 1: List of *Drosophila* stocks used in RNAi, Overexpression, and Gain of Function studies.

Bloomington Stock ID	**Flybase ID**	**Gene**	**Genotype**
N/A	FBst0036303	*Attp2*	*y^1 v^1; P{CaryP}attP2*
N/A	FBgn0264255	*Paralytic RNAi*	*w^{1118}; UAS-Para-RNai*
N/A	N/A	*Wild-type* (W1118)	*w^{1118}*
N/A	FBtp0039691	*Ppk-GAL4*	*w^{1118}; ppk-GAL4*
N/A	N/A	*Ppk-GAL4 UAS-Dicer 2*	*w^{1118}; ppk-GAL4; UAS-Dicer2*
#36820	FBst0036820	*Gαq RNAi*	*y^1 sc^* v^1; P{TRiP.GL01048}attP2*
#31268	FBst0031268	*Gαq RNAi*	*y^1 v^1; P{TRiP.JF01209}attP2/TM3, Ser^1*
#31113	FBst0031113	*NorpA RNAi*	*y^1 v^1; P{TRiP.JF01585}attP2*
#31197	FBst0031197	*NorpA RNAi*	*y^1 v^1; P{TRiP.JF01713}attP2*
#31243	FBst0031243	*PKA-c2 RNAi*	*y^1 v^1; P{TRiP.JF01756}attP2/TM3, Ser^1*
#31133	FBst0031133	*gi RNAi*	*y^1 v^1; P{TRiP.JF01608}attP2*
#31277	FBst0031277	*PKA-c1 RNAi*	*y^1 v^1; P{TRiP.JF01218}attP2*
N/A	N/A	*Tm6b Tb*	*w^{1118};Tm3, Sb/Tm6b, Tb*
#30734	FBst0030734	*Gαq* Overexpression	*w^*; P{UAS-Gαq.R}2*
#35529	FBst0035529	*NorpA* Overexpression	*w^{1118}; P{UAS-norpA.WT}2*
#30743	FBst0030743	*Gαq* Gain of Function	*P{UAS-Gαq.Q203L}F58a, w^*/FM7i, P{ActGFP}JMR3*

RNAi Screen:

A list of 26 candidate genes that were expected to play a role in the Gαq/PLC signaling transduction pathway were compiled by graduate student Mike Mutchler and tested via 38 *UAS-RNAi* lines that were ordered from Bloomington *Drosophila* Stock Center at Indiana University. The *GAL4/UAS* system was used to express RNAi in the *Drosophila. GAL4* can be used to activate transcription at the *Upstream Activation Sequence* (*UAS*), the *ppk-GAL4* driver produces *GAL4* expression and transcriptional activation in mdIV neurons. *Dicer 2* is a gene that has been shown to enhance the effectiveness of RNAi knockdown (Honjo, Mauthner, Wang, Skene, & Tracey, 2016). Thus, the genotype of the virgin female flies containing the *GAL4* driver used in this screen was *w; ppk-GAL4; UAS-Dicer2* which was crossed with males containing the *UAS* promoter located just upstream of the RNAi transgene. Interference RNA (RNAi) works through reverse complementation to the mRNA of a target gene. When the RNAi is transcribed it is cleaved by the enzyme coded by *Dicer 2,* producing short interfering RNA fragments (siRNA). This binds to and activates the RNA induced silencing complex (RISC) which binds to the mRNA of the targeted gene resulting in degradation and thus silencing of the target mRNA. The F1 generation of larvae were tested in thermal nociception assays at 46°C with sample sizes around 25 larvae per RNAi line.

RNAi Knockdown, overexpression, and Gain of Function Cross Compositions:

The protocol for setting up the RNAi knockdown was similar to that previously outlined by Caldwell et al. in a study which used *Drosophila* to identify novel genes involved in nociception (Caldwell, & Tracey, 2010) with a few modifications. Duplicates of six virgin *ppk-*

GAL4-Dicer 2 females were mated in a vial with three young males of the *UAS-RNAi* line. Three young male flies, #36303, containing no *UAS-RNAi* transgene were crossed with six female *ppk-GAL4 Dicer 2* virgins serving as the negative RNAi control. Three young male *Para RNAi* flies were crossed with *ppk-GAL4 Dicer 2* to act as a positive control. *Paralytic* (*Para*) is a gene in *Drosophila* that codes for the α–subunit of the sodium gated ion channels necessary for firing of neural action potentials (Warmke et al., 1997) in the mdIV neurons. The vials were then placed in an incubator at 25°C and ~70% humidity and transferred (flipped) to new vials on the third, fourth, and fifth days. This allowed for each vial of larvae to mature a day apart from each other enabling the thermal or mechanical testing of the larvae to be spaced out over multiple days. The same number of larvae were tested from each genotype on each day of testing to account for any environmental differences that may have occurred.

The protocol for setting up the overexpression cross was the same as that for RNAi except that the fly genotypes differed. In this cross, *ppk-GAL4* was used instead of *ppk-GAL4 Dicer 2*. Transcription of the *Dicer 2* gene helps enhance RNAi knockdown (Honjo et al., 2016) but is not necessary for overexpression. Instead of a *UAS-RNAi* line, *UAS*-overexpression lines (#30734 and #35529) were used to drive overexpression of cDNA sequences for *Gαq* and *NorpA* and thus increase translation of the protein that gene codes for. To test the overexpression, six female *ppk-GAL4* virgins were mated with three young males of the *UAS*-overexpression line in a vial. Additionally, 6 virgin *ppk-GAL4* females were crossed with three young W1118 (wild-type larvae that do not have a UAS-transgene) males as a control for the *GAL4*. To serve as a control for the *UAS*-overexpression lines three young males were mated with six female W1118 virgins, W1118 also lacks the driver necessary to express a *UAS*-transgene.

The protocol for setting up the gain of function cross (GoF) was the same as that used for the overexpression study except that a *UAS*-gain of function line (#30743) was used to express a mutant cDNA that has constitutive enzymatic activity for the gene of interest, *Gαq*. The controls were set up in the same fashion as mentioned in the overexpression experiment. Additionally, the scale of the GoF experiment was larger than the scale in other experiments with quadruple replicates of each genotype due to inherent lethality, further explained in the results section.

Thermal Nociception Assay:

The protocol for the thermal nociception assay follows that previously outlined by Caldwell et al. in a study which used *Drosophila* to identify novel genes involved in nociception (Caldwell, & Tracey, 2010). For the thermal assays larvae were tested when they reached the point in development known as wandering third instar. The larvae were washed from the food vials with distilled (DI) water onto a petri dish. A sprinkle of yeast was added to the dish and allowed to dissolve to reduce the surface tension of the water. Any food or larvae that were not third instar were removed and discarded. Excess water that was present in the dish was removed so the surface of the dish was still moist and the larvae were crawling but not swimming.

A soldering iron attached to a thermocouple and Variac Voltage controller was used to gently apply the thermal stimulus at 46°C (between 46.0 and 46.9°C) to either side of the larvae's midsection. The behavioral response was recorded using a video camera mounted to the microscope and the video was later analyzed using Adobe Premier to accurately record the start and stop times of NEL. Latency is the amount of time (seconds) that it takes the larvae to make one complete barrel-role in the NEL behavior after contact with the thermal stimulus. The start time was the point at which the thermal stimulus was applied and the stop time was recorded

when the larvae had made one complete rotation along its longitudinal axis. Start time was subtracted from stop time to yield the latency (sec) for each organism. The latency for all organisms of a genotype were compiled to determine average latency of that genotype. Statistical significance for the thermal assays was determined using a Mann-Whitney Rank Sum Test via the computer program, SigmaPlot.

Mechanical Nociception Assay:

The protocol for the mechanical assay was the same as that used in the thermal assay differing in only stimulus and recording of data. As outlined by Caldwell et al. (Caldwell, & Tracey, 2010) the mechanical stimulus was delivered as a quick poke on the dorsal midline of the larvae perpendicular to the longitudinal axis. The mechanical stimulus was delivered using an apparatus composed of a bent glass rod with an attached 10-mm Von Frey filament calibrated to deliver a 50 mN force to the larvae. The instant the filament bent, the probe was removed and behavior of the larvae was observed for ten seconds. The scoring system was binary; it was recorded that a larva either did or did not exhibit the NEL behavior. This data was aggregated over all samples tested per genotype, to yield the percent response for that genotype. Each larva was poked and scored three times with about ten seconds in between each poke but only the first poke per organism was used in the final analysis. Statistical significance for mechanical nociceptive assays was determined via Chi-squared tests using the program, SigmaPlot.

Results

There are multiple G protein signaling pathways that could be involved in sensory neuron function. The screen was intended to identify the most relevant pathways and genes. In this screen, 26 putative G protein signaling genes were systematically knocked down in the nociceptors to identify genes that produce a defect in nociception. The effects of these genes on thermal nociception were evaluated using latency to roll (sec). A genetic screen has smaller sample sizes than could yield significance yet a large enough sample size to yield trends. The screen shown below in Figure 1 involves the knockdown of the 26 putative G protein signaling genes via RNAi 38 lines to manipulate G protein signaling in the mdIV neurons.

**Note to reader: The enormous amount of work required to collect this data was largely done by a former graduate student of the Bellemer Lab, Mike Mutchler. I came in on the tail end of the screen and only collected data that accounts for about 10 percent of the data represented in Figure 1.

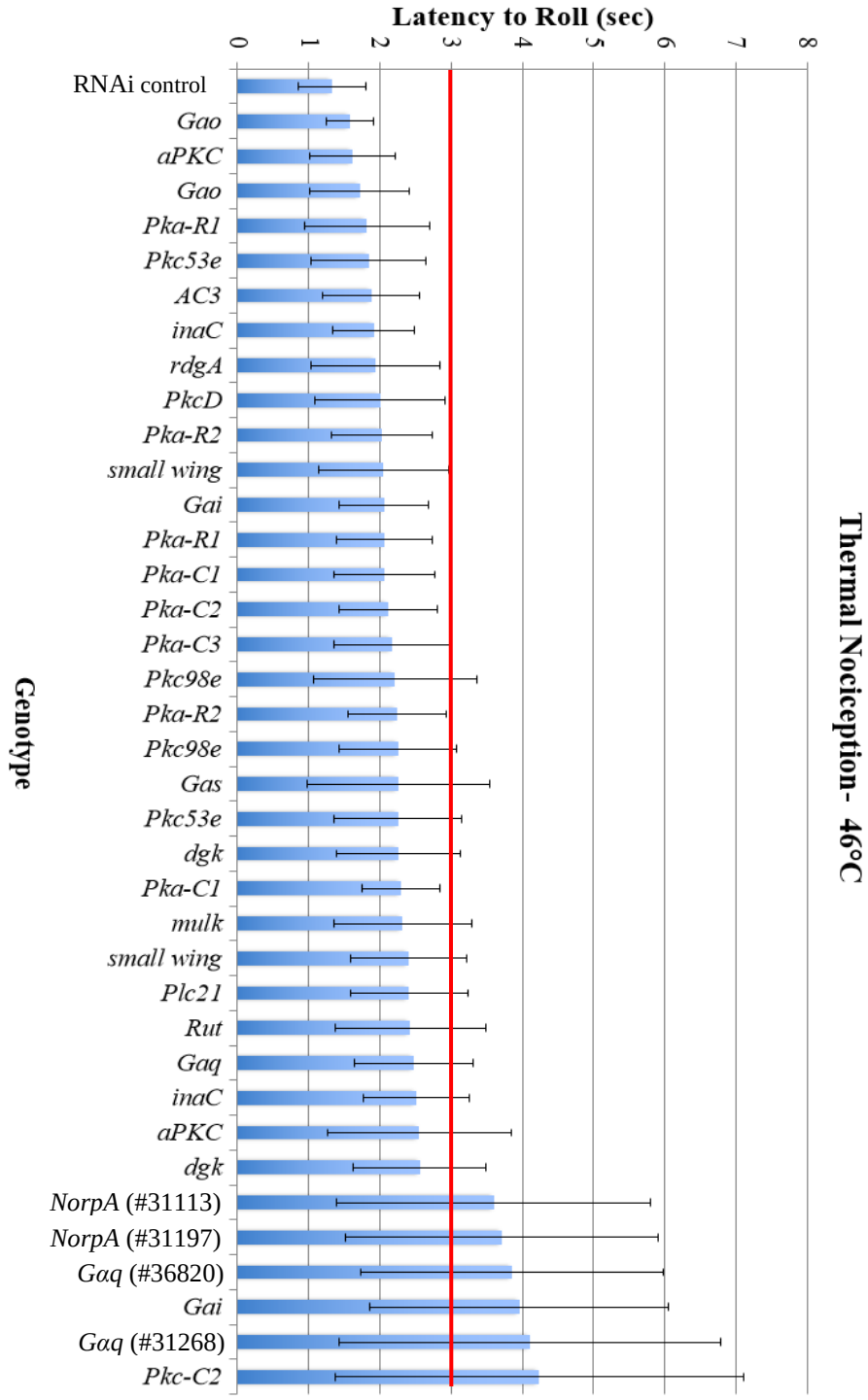

Figure 1: A preliminary RNAi knockdown screen of 38 RNAi lines targeting genes, involved in G protein signaling, identified six thermal nociception defective fly lines tested at 46 °C, cutoff 3 seconds, with RNAi genotypes, *NorpA* (x2), *Gαq* (x2), *Gai,* and *Pkc-c2*.
(n~25, error bars= standard deviation)

Of the 38 transgenic *Drosophila* lines screened in Figure 1, six of them appeared to yield a deficiency in thermal nociception at 46 °C representing four RNAi transgenes, *NorpA* (x2), *Gαq* (x2), *Gai,* and *Pkc-c2,* in relation to the response time elicited in RNAi control larvae. These flagged genes required further investigation and a mechanical nociception assay was performed on the four candidate genes identified in the screen. Appendix Figure A1 shows the results of this assay which identified RNAi knockdowns of *Gαq* and *NorpA* to yield a decrease in the proportion of animals responding to the mechanical stimulus, which leads us to believe that the animals are less sensitive to the stimulus. In the follow-up assay (Figure A1), RNAi knockdown of *Gai, PKA-c2,* and *PKA-c1* failed to yield a mechanical insensitivity phenotype in a larger sample (n=59-63) than the screen (n~25).

The observed increase in latency in response to thermal stimuli of *Gαq* and *NorpA* mutants lead us to believe that RNAi targeting these genes caused the strongest nociception phenotype, suggesting that these genes have the biggest role in nociception. To confirm this finding, we tested two different *Gαq RNAi* lines (#36820 and #31268) and two different *NorpA RNAi* (#31113 and #31197) lines for a defect in mechanical nociception with a sample size of 80 for each RNAi line pictured in Figure 2 and Figure 3.

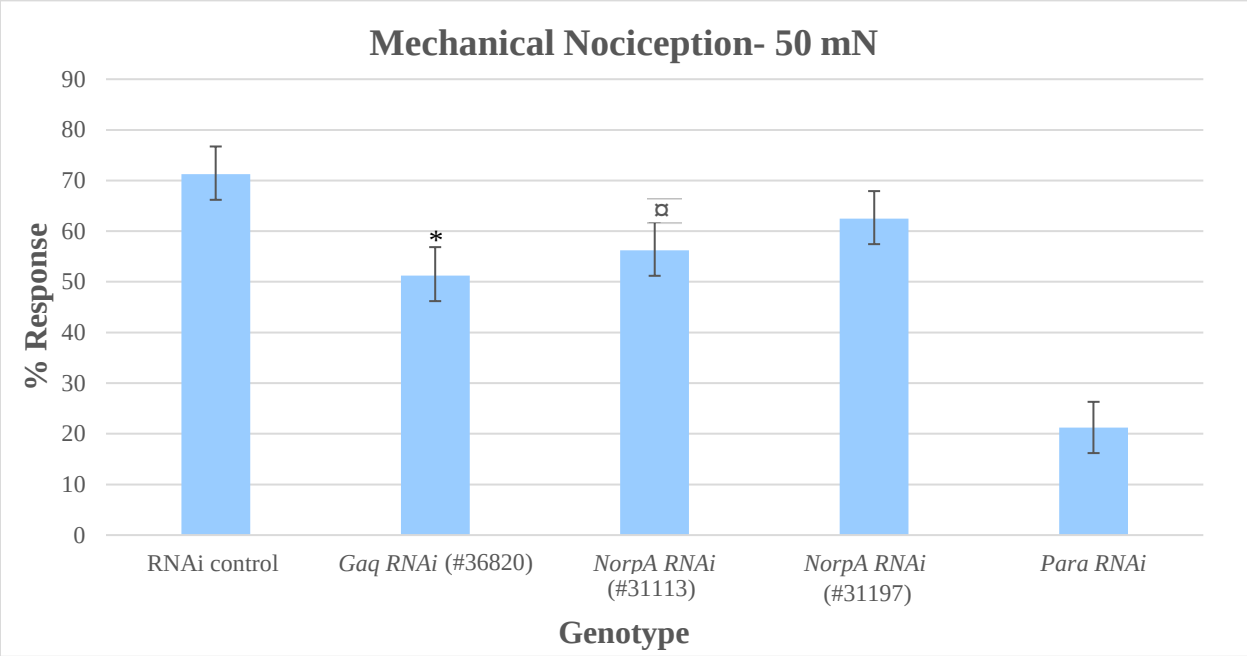

Figure 2: RNAi knockdown of *Gαq* in the nociceptor neurons produced a significant decrease in sensitivity to a ~50 mN mechanical stimulus, *NorpA RNAi* did not produce a significant decrease in sensitivity but trended towards significance.
(n=80 per genotype, *p<0.05, ¤p=0.07, error bars = standard error)

Figure 2 shows with significance, p<0.05, that RNAi knockdown of one *Gαq* line in mdIV neurons result in a defect in mechanical nociception compared to the RNAi control. RNAi knockdown of *NorpA* failed to show a significant decrease in sensitivity to noxious mechanical stimuli but trended strongly towards significance, p=0.07.

Figure 3 displays the mechanical nociceptive behavior for the second *Gαq* line (#31268) which had to be balanced due to lethality of the *UAS-RNAi* transgene. However, the *Tm6b Tb* crossed with the second *Gαq* line (#31268) allowed us to visually determine which chromosome the progeny inherited and test only those expressing *Gαq RNAi*.

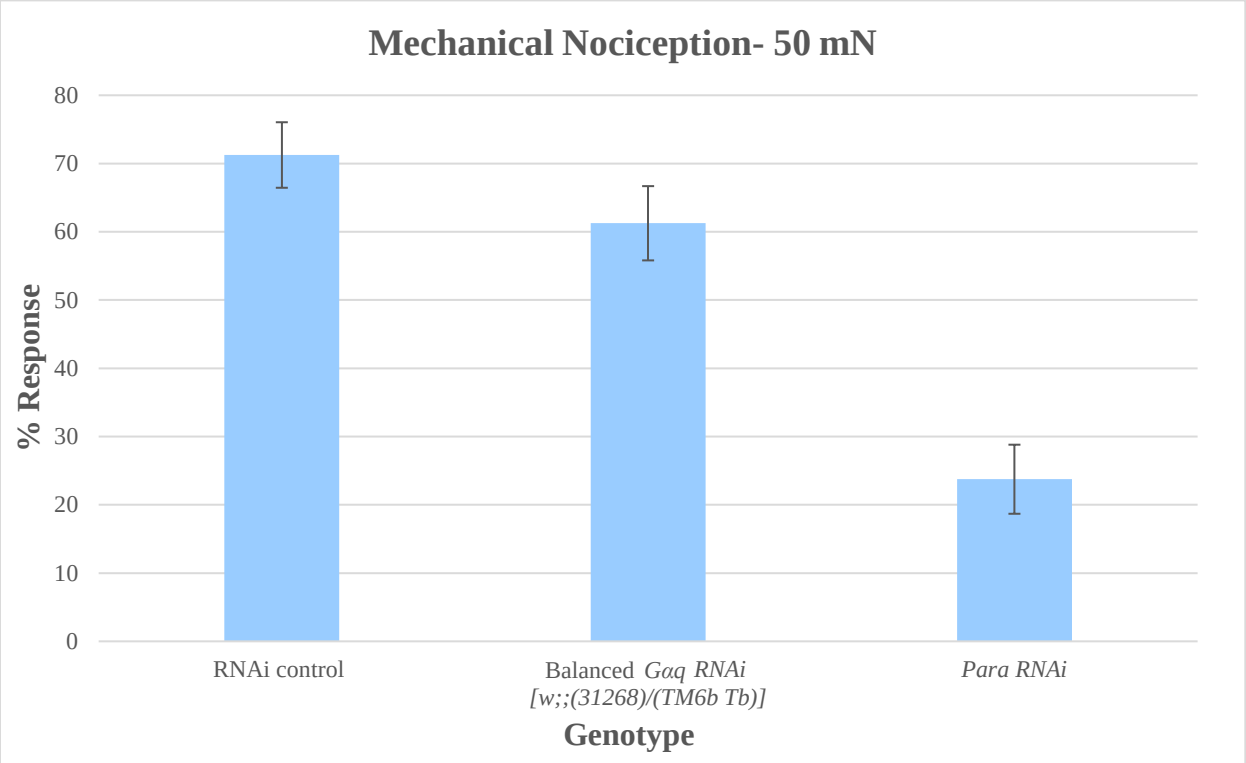

Figure 3: RNAi knockdown of *Gαq* in #31268 balanced with *TM6b Tb* in the nociceptor neurons failed to show a significant decrease in sensitivity to a ~50 mN mechanical stimulus. (n=80, p=0.242, error bars = standard error)

Figure 3 shows the mechanical data for the balanced *Gαq* line, #31268, which is heterozygous for the RNAi gene due to an inherent homozygous lethality. Mendelian inheritance would thus state that only half the balanced larval progeny would contain the RNAi while the other half of the larvae would be effectively wild-type. For this reason, a balancer, *TM6b Tb* was used so that the larvae containing the RNAi transgene could be visually identified and those larvae were tested. The result of this assay was that the balanced *Gαq* line, #31268, which coded for RNAi that knocked down *Gαq* function in the nociceptors, did not produce a significantly reduced sensitivity to noxious mechanical stimuli.

Figures 2 and 3 identified *Gαq* and *NorpA* to play an important role in mechanical nociception. The next step was to then mirror the same experiments in Figures 2 and 3 but utilize a thermal assay instead of a mechanical assay to see if *Gαq* and *NorpA* have similar roles in thermal and mechanical nociception. Figures 4 and 5 show the results of the thermal assays performed on the RNAi lines of *Gαq* and *NorpA*.

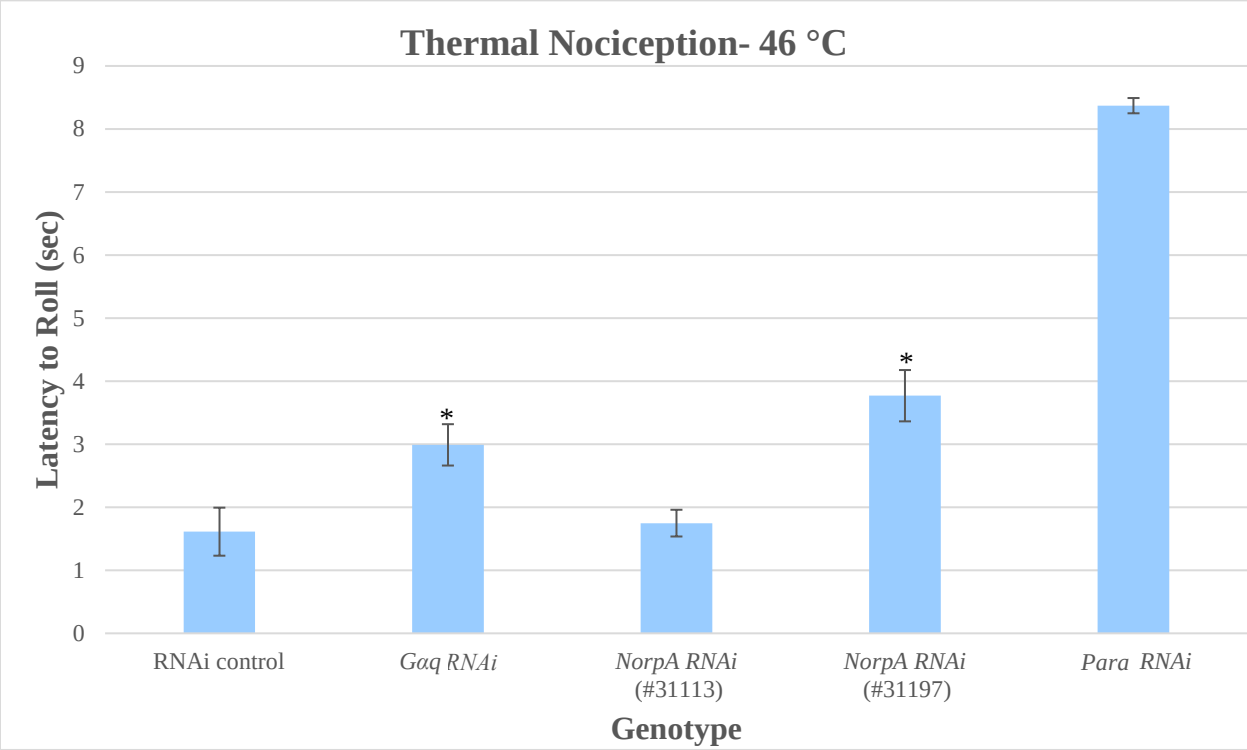

Figure 4: RNAi knockdown of *Gαq* and *NorpA* (31197) in the nociceptor neurons showed a significant decrease in sensitivity to a 46°C thermal stimulus.
(n=42-50, *p<0.001, error bars = standard error)

Figure 4 shows with a high degree of significance, p<0.001, that RNAi knockdown of one *Gαq* line (#36820) and one *NorpA* line (#31197) in mdIV neurons result in a defect in thermal nociception. This phenotype observed from silencing of *Gαq* and *NorpA* leading to increased latency and suggestive of decreased sensitivity in thermal nociception suggests that these proteins are necessary for normal mechanosensation. Interestingly, one *NorpA* line, #31113,

which produced a significant decrease in sensitivity to mechanical stimuli in Figure 2, did not result in a significant decrease in sensitivity to thermal stimuli.

Figure 5 displays the thermal nociceptive behavior for the second *Gαq* line (#31268) which again, had to be balanced because the *UAS-RNAi* transgene caused lethality.

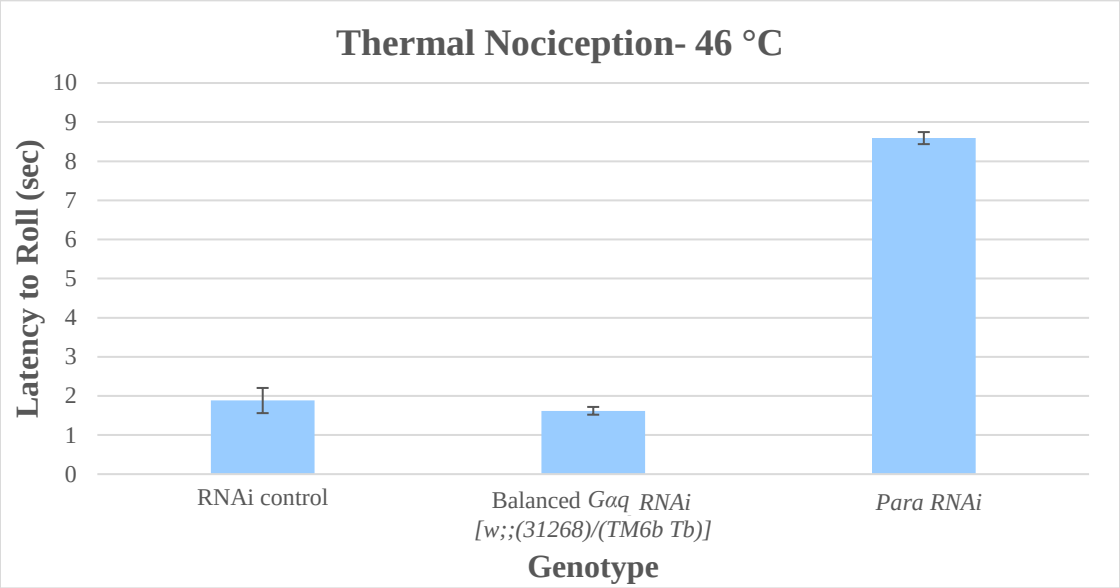

Figure 5: RNAi knockdown of a *Gαq* in #31268 balanced with *TM6b Tb* in the nociceptor neurons failed to show a significant decrease in sensitivity to a 46°C thermal stimulus. (n=46-50, p=0.671, error bars = standard error)

Figure 5 shows the thermal data for the balanced *Gαq* line, #31268, which was balanced with *TM6b Tb*. The result of this assay was that the balanced *Gαq* line, #31268, did not produce a significantly reduced sensitivity to noxious thermal stimuli and had a slightly lower average latency than that of the wild type RNAi control. These results are consistent with those found in the mechanical assay and suggest that there may be an issue with the expression of the RNAi in this line.

The findings in Figures 2-5 show that the silencing of *Gαq* and *NorpA* with RNAi lead to a decrease in sensitivity in both mechanical and thermal nociception. This suggests that they are both necessary for normal nociception and thus the knockdown of such resulted in a significant phenotype. The next step was to perform an overexpression study and see if higher expression of *Gαq* and *NorpA* resulted in an increased sensitivity to nociceptive stimuli. This result would be expected if *Gαq* and *NorpA* work to increase nociceptor sensitivity.

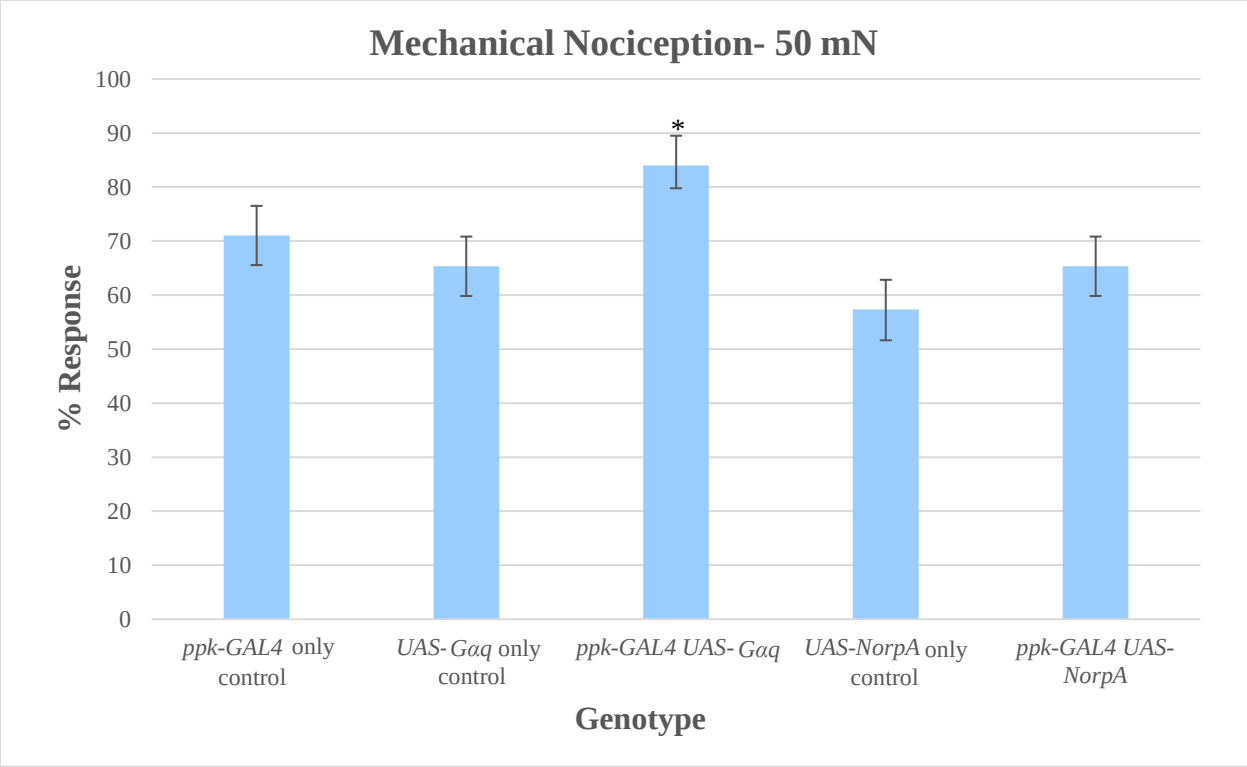

Figure 6: Overexpression of *Gαq* and *NorpA* in the nociceptor neurons produced a significant increase in sensitivity to the ~50 mN mechanical stimuli in the *Gαq* overexpression. However, a significant increase in sensitivity was not observed in the *NorpA* overexpression. (n=69-75, *p=.030, error bars = standard error)

Figure 6 displays the data for an overexpression study of *Gαq* and *NorpA*. The *ppk-GAL4*, *UAS-Gαq* only, and the *UAS-NorpA* only lines were used to show that driver itself (*GAL4*) and the overexpression lines (*UAS-Gαq* only, and *UAS-NorpA* only) did not cause a significant

phenotype. The overexpression of *Gαq* yielded a significant increase ($p<0.05$) in sensitivity to mechanical stimuli over the *ppk-GAL4* only line. The overexpression of *NorpA* did not show a significant increase in sensitivity to mechanical stimuli.

In follow-up to the results found in Figure 6, the final assay performed in this study was a gain of function experiment which would be expected to yield similar results to overexpression through constitutive activation of the cDNA which would be expected to yield an even stronger phenotype than overexpression. The data for this thermal nociception assay can be seen in Figure 7.

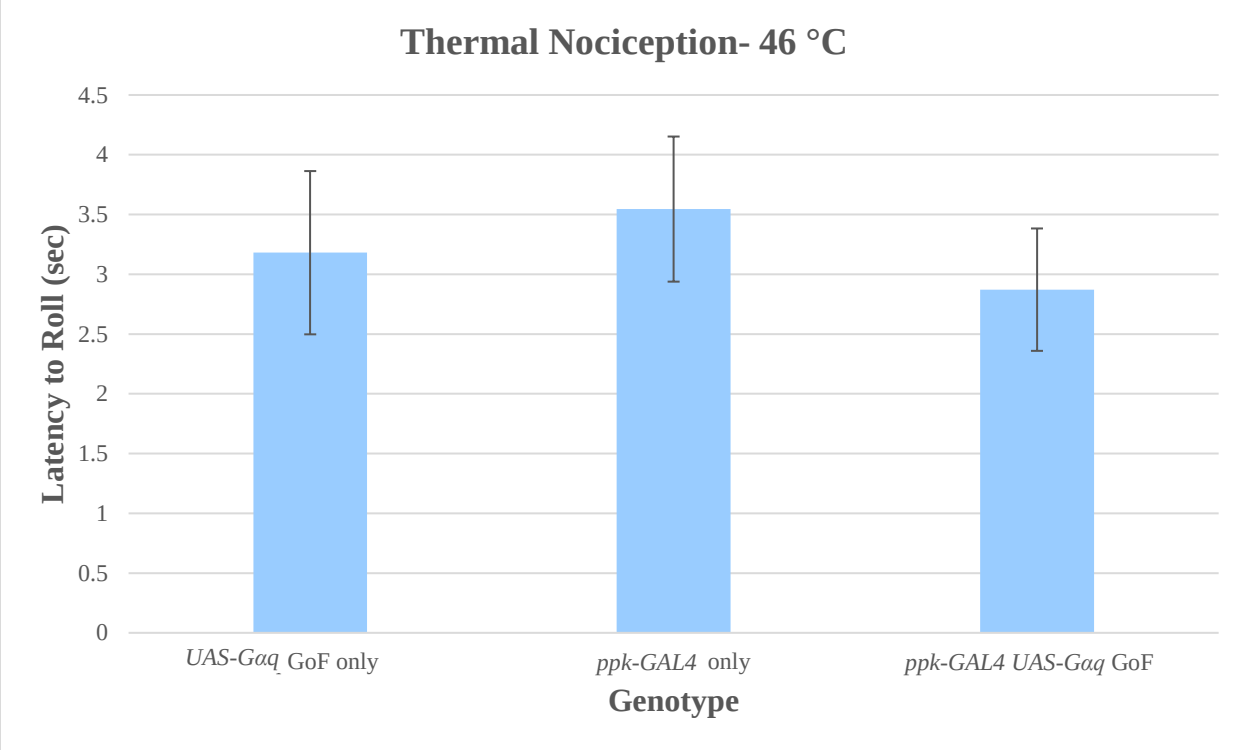

Figure 7: The *Gαq* gain of function study failed to yield a significant increase in sensitivity, but appeared to trend in that direction. The *Gαq* gain of function larvae had an increased rate of lethality and thus only a small sample was tested in a thermal nociception assay at 46 °C. (n=21-26, $^{NS}p=0.820$, error bars= standard error)

Figure 7 shows the data for the gain of function experiment in response to a thermal stimulus. It was discovered during the conduction of this experiment over multiple attempts that crosses containing the *UAS-Gαq* gain of function transgene produced very few viable progeny, because the gain of function of *UAS-Gαq* has a high rate of lethality associated with it. This lethality made it impossible to get a high enough sample size to determine any level of significance. Despite the lack of significance, it can be said that the data trends in a manner that suggests if the sample size were higher, gain of function for *Gαq* would result in lower latency and thus increased sensitivity to noxious heat.

Discussion

A reduction in sensitivity to thermal nociception was observed in the knockdown of two genes, *Gαq* and *NorpA*, involved in the G protein signaling pathway. A reduction in sensitivity to mechanical nociception was observed in the knockdown of *Gαq* and with *NorpA RNAi* trending towards significance. Additionally, the overexpression study showed an increased sensitivity to noxious stimuli when *Gαq* was overexpressed. This data demonstrates that *Gαq* and *NorpA* are necessary for normal thermal nociception and that *Gαq* is necessary for normal mechanical nociception in *Drosophila*.

Gαq is a subunit of the heterotrimeric G proteins in *Drosophila* composed of alpha, beta, and gamma subunits that are activated via exchange of GDP for GTP. When GTP binds, the alpha subunit (Gα) dissociates from the beta and gamma dimer. The Gα subunit is then able to interact with its downstream effectors and play a role in many aspects of cellular signaling. This study established NorpA (PLC-β) to be the most substantial downstream effector. Figure 8 below is a schematic depicting the proposed role that Gαq and NorpA have in a pain signaling pathway.

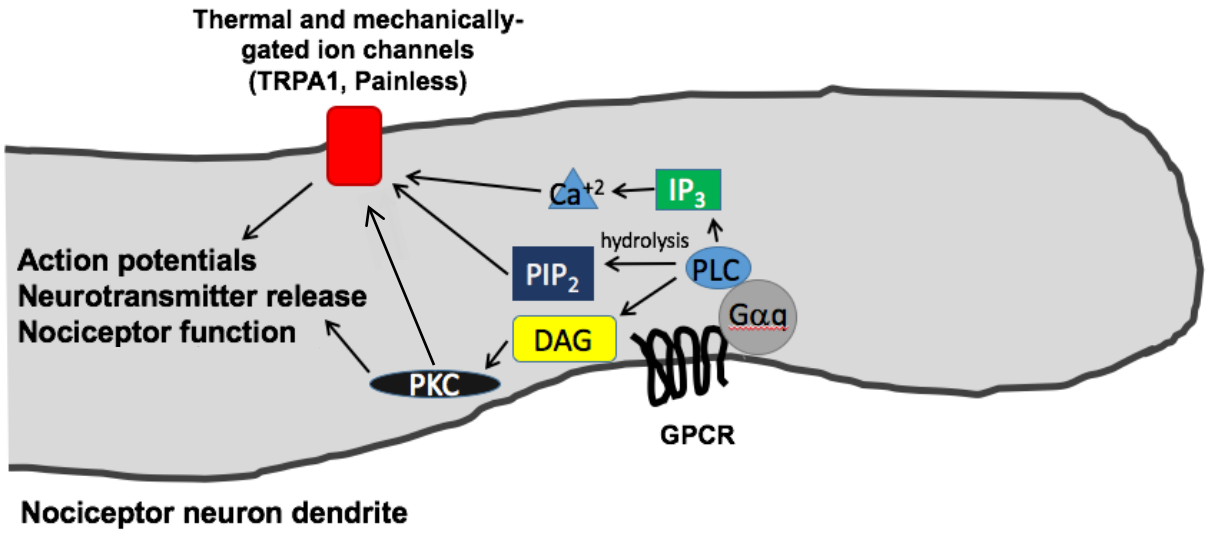

Figure 8: A proposed signaling pathway for G protein and PLC-β mediated nociception in *Drosophila.*

When a GPCR is activated by a ligand it allows for a conformational change that enables the heterotrimeric G protein to be activated by GTP. When this happens, GDP dissociates from the Gαq subunit and the higher energy GTP binds to the Gαq subunit in the place of GDP. The Gαq subunit is then considered to be activated and can interact with a host of downstream effectors. Of these effectors, PLC-β is the most substantial as it pertains to nociception. After Gαq activates PLC-β, it hydrolyzes the GTP bound to the Gαq subunit to GDP and Gαq returns to the inactive state and reassociates with the beta/gamma dimer (McCudden et al., 2005). PLC-β plays a critical role in the mediation of signaling molecules such as PIP_2, IP_3, and DAG. PLC-β catalyzes the hydrolysis of PIP_2 which allows for higher levels of calcium to be present within the neuron as PIP_2 reduces intracellular Ca^{2+} release. IP_3 and DAG are the products of the hydrolysis of PIP_2, the presence of IP_3 is known to stimulate intracellular Ca^{2+} levels. The release of intracellular calcium is substantial because Ca^{2+} has been demonstrated to play a direct role in the activation of multiple TRP family cation channels (Rohacs, 2014) and would ultimately contribute to membrane depolarization and neural firing of the nociceptors.

Clapham et al. conducted a study which demonstrated that Ca^{2+} ions have a role in activating some TRP channels in mice through direct TRP-Ca^{2+} binding (Clapham, 2003). PLC also influences DAG which plays a role on the gating of TRP channels and nociceptor function through phosphorylation by PKC. Hofmann et al. conducted a study which demonstrated that DAG was also shown to activate TRP channels in humans independent of PKC and PLC-β activity, stating that DAG itself can activate ion channels via activation of non-selective cation currents (Hofmann et al., 1999). TRPA1 in *Drosophila* is a nonspecific cation channel so there is reason to believe that it too may be directly activated by DAG, although to my knowledge, this has not yet been explicitly shown. DAG has also been demonstrated to play a central role in

neurotransmitter release, another method of TRP channel activation, as it pertains to synaptic vesicle cycling in mice (Tu-Sekine, Goldschmidt, & Raben, 2015). This is the mechanism by which we propose *Gαq* and *NorpA* to play a role on thermal and mechanical nociception.

Some data presented in study differs from what was expected, the first instance is the data depicted in Figure 2. In this figure a significant decrease in sensitivity to noxious mechanical stimuli was expected to be seen in the knockdown of both *Gαq* and *NorpA.* However, a significant decrease in sensitivity was only seen in the *Gαq RNAi* larvae while the *NorpA RNAi* larvae trended towards significance. An increased sample size would have been likely to provide enough significance to demonstrate *NorpA* to play a role in the normal mechanical nociception.

In Figures 3 and 5 a significant decrease in sensitivity to mechanical and thermal noxious stimuli was expected to be seen in the *Gαq* knockdown. However, the *Gαq* knockdown failed to show a decrease in sensitivity for noxious thermal or mechanical stimuli thus, the expected phenotype was not produced. Perkins et al. conducted a study which demonstrated that that RNAi knockdowns are not always effective (Perkins et al., 2015). We propose that this was the case with the *Gαq RNAi* transgene in the line, #31268. Low levels or no transcription of the *Gαq RNAi* transgene expressed in #31268 would have resulted in the observed phenotype for this line. Future investigation of a third *Gαq RNAi* line to act as a definitive “tie breaker” for the role of *Gαq* knockdown in nociception would be useful.

Figure 2 displays data showing that neither of *NorpA RNAi* lines yielded a significant decrease in response rate, indicative of insensitivity, to mechanical stimuli. However, in Figure 4, one of the *NorpA RNAi* lines (#31197) produced a significant increase in latency, indicative of a decreased sensitivity to noxious thermal stimuli. One of the *NorpA RNAi* lines (#31113) in Figure 2 did however trend strongly towards significance with p=0.07. Based on this being just

outside of the statistically significant threshold, $p<0.05$ and the role that *NorpA* has been demonstrated to play in thermal nociception, it is likely that with an increased sample size we would have demonstrated *NorpA* to play a role in the normal detection of noxious mechanical stimuli.

Figure 6 depicts the data for the overexpression of *Gαq* and *NorpA cDNA*. It was expected that the larvae overexpressing both *Gαq* and *NorpA* would yield an increased response and subsequently indicate an increase in sensitivity to noxious thermal heat. Instead, the data yielded in this experiment displayed the expected phenotype of increased response rate and thus sensitivity to noxious thermal stimuli for the *Gαq* overexpression but failed to show an increased response to noxious thermal stimuli in larvae overexpressing *NorpA*. Im et al. conducted a study which demonstrated that the overexpression of *tachykinin receptor* (*dTKR*) in *Drosophila*, found there to be an increased sensitivity to noxious thermal stimuli dependent on multiple G protein subunits including Gαq (Im et al., 2015). It is worth pointing out that the *UAS-NorpA* only larvae, the *UAS-NorpA* control, had a percent response that was nearly fifteen percent lower than that of the *ppk-GAL4* only larvae and that the *NorpA* overexpression did yield a higher percent response than that of the *UAS-NorpA* only larvae. Based on this observation, it is apparent that the *UAS-NorpA* overexpression transgene influences mechanical nociception that is independent of *GAL4* or overexpression, additional investigation would be required to identify the mechanism responsible. However, as the nociceptive response of the *NorpA* overexpression was compared to *ppk-GAL4* only, no significant increase in sensitivity to the noxious mechanical stimulus could be determined.

Finally, Figure 7 presents the data for a Gain of Function experiment of *Gαq*. This experiment expressed the constitutively active cDNA for *Gαq* and thus would have been

expected to yield an even more robust phenotype than the overexpression experiment in Figure 6. However, a high level of lethality in the larvae of the GoF study made it difficult to obtain enough testable larvae to provide any level of significance. For this reason, the data presented in Figure 7 cannot be used to make any conclusions as the larval sample size was only 21-26. However, it can be said that the data trended as expected with the *Gαq* GoF having the lowest average latency, suggestive of the potential for increased sensitivity to noxious thermal stimuli. The observed lethality is likely due to "leaky" overexpression of *Gαq*. This means that *Gαq* was probably overexpressed outside of the target mdIV neurons which, due to the wide range of functions for G proteins, could cause significant developmental issues in these larvae with lethal consequences. A GoF experiment of even larger scale, able to achieve a n of 45-50 would likely be required to determine any level of significance.

Future directions of study for the role that *Gαq* and *NorpA* play in thermal and mechanical nociception would be to investigate another *Gαq RNAi* knockdown line. This would further increase the confidence that *Gαq* does play a necessary role in nociception. It would also be useful to increase the sample size for the mechanical assay of the *Gαq* and *NorpA* knockdown experiments to determine a significant decrease in sensitivity to noxious mechanical stimuli. To display that the RNAi knockdown of *Gαq* and *NorpA* is responsible for the previously observed decrease in sensitivity to noxious stimuli, we could perform a rescue experiment in which we incorporate *Gαq* and *NorpA* genes from a related species of *Drosophila* in addition to the RNAi. This should serve the function of having *Gαq* and *NorpA* transcripts that would be similar enough to rescue the function of these genes in the larvae, yet have different enough mRNA that the RNAi is unable to degrade it.

Additionally, to observe a more robust phenotype in the overexpression studies, it may be useful to analyze *Gαq* and *NorpA* overexpression larvae for thermal hypersensitivity at 42°C instead of 46°C as done in this study. To determine whether the knockdown of *Gαq* and *NorpA* result in any differences in dendrite morphology, it would be useful to perform confocal microscopy on the RNAi knockdown of *Gαq* and *NorpA*. The data from morphological analysis of dendrites could be used to prove that *Gαq* and *NorpA* play a role in nociception through signal transduction rather than development of morphological anomalies. A final future direction of study would be to utilize Ca^{2+} imaging in RNAi knockdown larvae of *Gαq* and *NorpA* to analyze Ca^{2+} influx in the dendrites of larvae under a noxious stimulus. We could also test the role that Ca^{2+} has on this signal transduction pathway by testing mutants of the IP_3 receptor. This could help support the previously proposed mechanism in which *Gαq* and *NorpA* are necessary to normal nociception.

In conclusion, this study identified *Gαq* and *NorpA* out of a list of 38 RNAi knockdown lines, knocking down 26 different genes, to play the most critical role in thermal and mechanical nociception. We were able to confirm that *Gαq* and *NorpA* are both necessary for normal thermal nociception while *Gαq* is necessary for normal mechanical nociception in *Drosophila*. This role was confirmed via thermal and mechanical assays performed in both RNAi knockdown and overexpression experiments.

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Appendix:

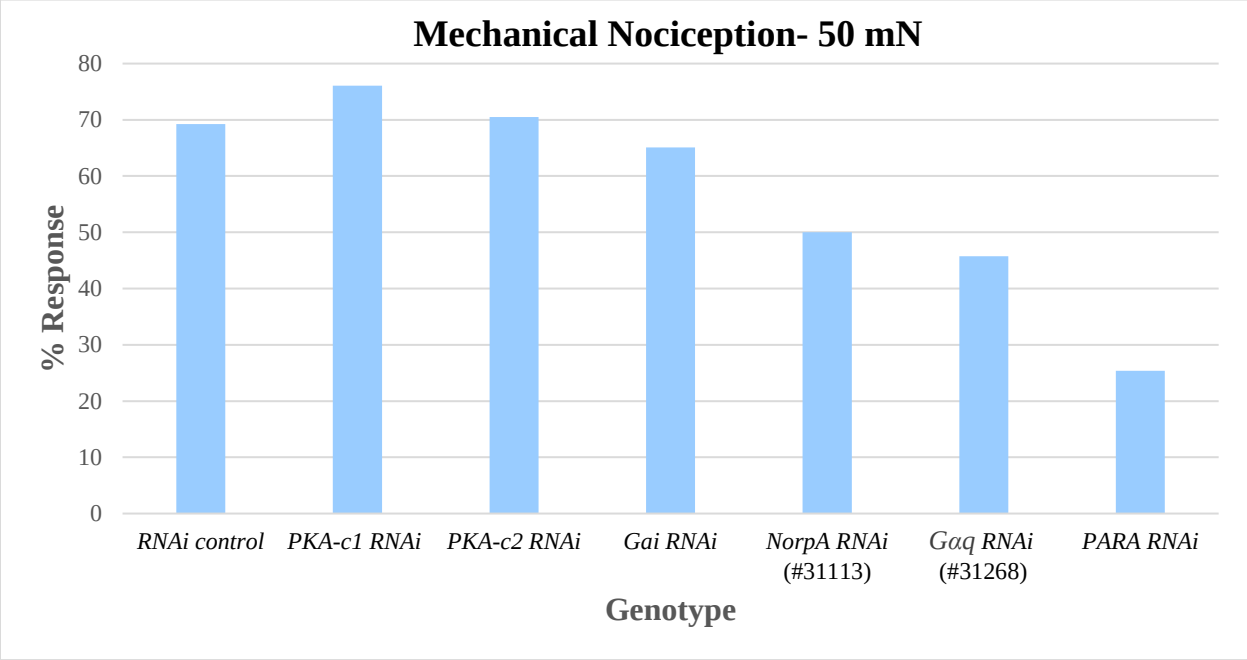

Figure A1: Initial follow-up data for the screen shown in Figure 1. This identified more concretely which genes produced a significant phenotype of decreased sensitivity when knocked down, these results have some overlap with Figure 2 and were not deemed necessary to include in results but is consistent with the results seen in Figure 2.

(n=59-63)